

NEURON-RESTRICTIVE SILENCER FACTOR PROTEINS

FIELD OF THE INVENTION

The present invention relates to neuron-restrictive silencer factor proteins, nucleic acids, and antibodies thereto.

BACKGROUND OF THE INVENTION

The molecular basis of neuronal determination and differentiation in vertebrates is not well understood. In other lineages, systematic promoter analysis of cell-type specific genes has led to the identification of genetically essential transcriptional regulators of lineage determination or differentiation

10 L.M. Corcoran, et al., *Genes and Development* 7, 570-582 (1993); S. Li, et al., *Nature (London)* 347, 528-533 (1990); L. Pevny, et al., *Nature* 349, 257-260 (1991). To apply this approach to the development of neurons, the transcriptional regulation of a neuron-specific gene, *SCG10*, has been previously examined (D.J. Anderson, R. Axel, *Cell* 42, 649-662 (1985).

15 *SCG10* is a 22 Kd, membrane-associated phosphoprotein that accumulates in growth cones and is transiently expressed by all developing neurons (R. Stein, N. Mori, K. Matthews, L.-C. Lo, D.J. Anderson, *Neuron* 1, 463-476 (1988); U.K. Shubart, M.D. Banerjee, *J. Eng. DNA* 8, 389-398 (1989)). Upstream regulatory sequences controlling *SCG10* transcription have been

transcribed from a λ H1 fusion construct. The probe was a radiolabeled restriction fragment containing two tandem copies of S36. Competitors used were the S36, Na33 and Sm36 oligonucleotides and an oligonucleotide containing an Ets factor binding site (Ets) (22). The large arrowhead marks the λ H1 encoded protein DNA complex (lane 1), the small arrowhead marks the NRSF:DNA complex (lane 9). No complexes were formed by an *in vitro* translation reaction to which no RNA had been added (data not shown).

10 ^{ins D2} Figures 4A and 4B show that antibodies against GST- λ H1 recognize the native NRSF:DNA complex. ^{ins D3} (A) The indicated amounts (in μ l) of α GST- λ H1 ascites (48) or a control ascites were added to a mobility shift reaction containing HeLa nuclear extract. The competitor was the S36 oligonucleotide present at 300 fold molar excess. The bracket indicates the supershifted NRSF:DNA complex, and the small arrowhead marks in the NRSF:DNA complex. ^{ins D4} (B) A mobility shift reaction using a rabbit reticulocyte reaction programmed with λ -H1 encoding RNA. The mobility shift reactions were preformed and analyzed as in the upper panel. For supershift experiments, ascites fluid was included during this incubation. The reactions were performed as in Fig. 3, except that the acrylamide gel used for analysis had an 80:1 acrylamide to bis ratio instead of 30:0.8. The bracket indicates the supershifted λ H1-encoded protein: DNA complex, and the large arrowhead marks the λ H1-encoded protein:DNA complex. Attempts to obtain an quantitative supershift using higher concentrations of antibody were precluded by the inhibition of DNA binding that occurred when the amount of ascites in the SMSA was increased.

25 Figure 5 shows that native and recombinant NRSF recognizes NRSE in four different neuron-specific genes. Electrophoretic mobility shift assays were preformed using either nuclear extract from HeLa cells (lanes 1-4), to reveal

the activity of native NRSF, or using *in vitro* synthesized NRSF encoded by the λ H1 cDNA (lanes 5-8). The labeled probes consisted of restriction fragments containing NRSEs derived for the rat SCG10 gene (SCG10, lanes 1-5); the rat type II sodium channel gene (NaCh, lanes 2 and 6); the human synapsis I gene (Syn, lanes 3 and 7) or the rat brain-derived neurotrophic factor gene (BDNF, lanes 4 and 8). The large arrowhead indicates the specific co-lex obtained with recombinant NRSF; small arrowhead that obtained with native NRSF. Note that the complexes obtained with all four probes are of similar sizes. The complexes obtained using HeLa extracts were partially supershifted with antibody to recombinant NRSF (cf. Fig.4)(data not shown).

Figure 6 depicts the nucleotide and deduced amino acid sequence of a partial cDNA (λ HZ4) for human NRSF (49). The nucleotide sequence is numbered in standard type, and the amino acid sequence in italics. The eight zinc fingers are underlined.

Figures 7A and 7B. (A) Schematic diagram of the predicted amino acid sequences from the NRSF cDNA clones. λ H1 is the original cDNA isolated by screening the HeLa expression library. λ HZ4 was isolated by hybridization to λ H1. (B) Alignment of NRSF zinc finger and interfinger sequences. The eight zinc fingers of human NRSF were aligned beginning with the conserved aromatic residue and including the interfinger sequences of fingers z2-7. The consensus for GLI-Krüppel zinc fingers and interfinger sequences is shown for comparison. The conserved tyrosine residue is boxed.

Figures 8A and 8B show the repression of transcription by recombinant NRSF. (A) A representative autoradiogram CAT enzymatic assays from

Figures 10A, 10B, 10C and 10D depict the comparison of NRSF and SCG10 mRNA expression by *in situ* hybridization. Adjacent transverse sections of E12.5 (A,B) and E13.5 (C,D) mouse embryos were hybridized with NRSF (A,C) or SCG10 (B,D) antisense probes. The arrows (A-D) indicate the ventricular zone of the neural tube. The large arrowheads (A-D) indicate the sensory ganglia and the small arrowheads, the sympathetic ganglia (C and D). Control hybridization with NRSF sense probes revealed no specific signal (Fig. 9C and data not shown).

Figures 11A, 11B and 11C depict the widespread expression of NRSF mRNA in non-neural tissues. *In situ* hybridization with an NRSF antisense probe (A,B) was performed on parasagittal sections of an E13.5 mouse embryo. (A) The arrowheads mark two positive tissues, the lung and the kidney; the arrow indicates the liver, which expresses much lower levels of NRSF mRNA (see also Fig. 9). (B) The arrowhead marks the ventricular zone in the telencephalon, the arrow indicates the heart. (C) An adjacent section to (B) was hybridized with an NRSF sense probe as a control for non-specific staining (59).

Figures 12A and 12B depict the nucleotide and deduced amino acid sequence of the complete cDNA for human NRSF. The nucleotide sequence is numbered in standard type, and the amino acid sequence in italics. The eight zinc fingers are underlined.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides neuron-restrictive silencer factor (NRSF) nucleic acids and proteins. The NRSF proteins of the invention silence or suppress the expression of neuron-specific genes. Without being bound by theory, it

SCG10 and NaII channel genes, respectively. Both of these elements have previously been shown to be sufficient to confer silencing activity and are bound by NRSF. The Sm36 sequence contains two point mutations in the S36 sequence and has an approximately 100 fold lower affinity for NRSF.

5 The sequence of the top strand of the oligonucleotides used for library screening and EMSAs are given below. The upper case sequences represent actual genomic sequence, the lower case sequences are used for cloning purposes.

S36: agctGCAAAGCCATTTCAGCACCCACGGAGAGTGCCTCTGC

10 Na33: ageATTGGGTTTCAGAACCACGGACAGCACCAGAGT

Syn: ageTTATGCCAGCTTCAGCACCCGCGGACAGTGCCTTCCA

BDNF: ageTTAGAGTCCATTTCAGCACCTTGGACAGAGCCAGCGGa

Ets: ageTTGCGGAACGGAAGCGGAAACCGa

(SEQ ID NO: 51)

(SEQ ID NO: 52)

(SEQ ID NO: 53)

(SEQ ID NO: 54)

(SEQ ID NO: 55)

15 Positive plaques from this screen were tested further for sequence specific DNA-binding by an additional screen with probes containing the SCG10 NRSE S36 or the mutated NRSE, Sm36 (15) N. Mori, S. Schoenherr, D.J. Vandenberg, D.J. Anderson, *Neuron* 9, 1-10 (1992). One phage was identified, λH1, that like native NRSF bound both the S36 and the Na33 probes but not the control Sm36 probe.

20 As an additional test of the authenticity of the cDNA clone, the DNA-binding specificity of its encoded protein was compared to that of native NRSF present in HeLa cell nuclear extracts using an electrophoretic mobility shift assay (EMSA). To generate recombinant protein, the λH1 insert was subcloned into the EcoRI site of pRSET B (Invitrogen), which provided an
25 in-frame start codon, a poly-histidine tag, and a T7 promoter, Recombinant λH1 was produced by *in vitro* transcription from linearized plasmid and *in vitro* translation using a rabbit reticulocyte lysate according to manufacturer's